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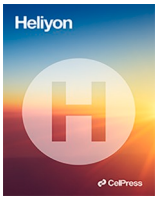

Diagnostic performance and longitudinal analysis of fungal biomarkers in COVID-19 associated pulmonary aspergillosis

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ABSTRACT

Objectives: Galactomannan lateral flow assay (GM-LFA) is a reliable test for COVID-19 associated pulmonary aspergillosis (CAPA) diagnosis. We aimed to assess the diagnostic performance of GM-LFA with different case definitions, the association between the longitudinal measurements of serum GM-ELISA, GM-LFA, and the risk of death.

Methods: Serum and nondirected bronchial lavage (NBL) samples were periodically collected. The sensitivity and specificity analysis for GM-LFA was done in different time periods. Longitudinal analysis was done with the joint model framework.

Results: A total of 207 patients were evaluated. On the day of CAPA diagnosis, serum GM-LFA had a sensitivity of 42 % (95 % CI: 23–63) and specificity of 82 % (95 % CI: 78–84), while NBL GM-LFA had a sensitivity of 73 % (95 % CI: 45–92), specificity of 85 % (95 % CI: 76–91) for CAPA. Sensitivity decreased through the following days in both samples. Univariate joint model analysis showed that increasing GM-LFA and GM-ELISA levels were associated with increased mortality, and that effect remained same with serum GM-ELISA in multivariate joint model analysis.

Conclusion: GM-LFA, particularly in NBL samples, seems to be a reliable method for CAPA diagnosis. For detecting patients with higher risk of mortality, longitudinal measurement of serum GM-ELISA can be useful.

1. Introduction

Several reports of COVID-19-associated pulmonary aspergillosis (CAPA) have been published as case series and observational studies [1–3]. Besides being associated with high mortality in intensive care units (ICU), it is challenging to diagnose CAPA [4]. The consensus statement of the European Confederation for Medical Mycology and International Society for Human and Animal Mycology (ECMM/ISHAM) requires at least one imaging characteristic (i.e., pulmonary infiltrates), one clinical characteristic (i.e., refractory fever, chest pain) and one mycological criterion for the CAPA diagnosis [5]. Because the first two requirements (imaging and clinical)

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are poorly specific and quite frequent in severe COVID-19 patients, CAPA diagnosis mainly relies on mycological criteria.

Since CAPA is a fatal health condition, diagnosis can be sped up with a point-of-care test, such as a lateral flow device [6]. As determined by a meta-analysis, its performance generated pooled sensitivity and specificity of 68 % and 87 % in serum and 86 % and 93 % in bronchoalveolar lavage (BAL) fluid samples, respectively [7]. The ECMM/ISHAM criteria now recognize *Aspergillus* Galactomannan Lateral Flow Assay (GM-LFA) as a mycological evidence [5]. However, most of the studies evaluated the accuracy of GM-LFA in patients with clinical suspicion of CAPA, not for screening purposes. Therefore, more data is needed to define the roles of these biomarkers in clinical settings.

Since joint models were proposed in 1997, they have been used in medical research for longitudinal data analysis [8–12]. Despite the abundance of data that could be analyzed more efficiently using these models, their usage is still noticeably sparse in clinical studies. These models combine longitudinal and time-to-event data into a single model, allowing for the inference of the dependence and association between the longitudinal biomarker and time-to-event data. Unlike traditional models, which treat these two data types separately with separate and independent densities, the joint model considers conditional joint density.

In this cohort, we aimed to screen the ICU population with GM-ELISA and GM-LFA for CAPA, starting a week later from the COVID-19 diagnosis, and assess the diagnostic performance of serum and nondirected bronchial lavage (NBL) LFA using the ECMM/ISHAM criteria in different time points. We also aimed to assess the prognostic value by evaluating the strength of the association between mort and the longitudinal measurements of serum GM ELISA and serum GM LFA collected during the study period with the joint model (JM) framework.

2. Materials and methods

2.1. Study design and data collection

Patients followed in COVID-19 ICUs between November 18, 2020, and April 24, 2021, were enrolled. It was designed as a prospective cohort study, and patients with a confirmed molecular diagnosis of SARS-CoV-2 admitted to the ICU because of acute respiratory failure were included. A total of 77 patients with previous IPA history (n = 3), neutropenia (n = 2), refractory shock (n = 2), those hospitalized in ICU for indications other than respiratory failure (n = 8), SARS-CoV-2 negative (n = 60), and who did not want to give consent (n = 2) were excluded [13]. Demographic data, body mass index, comorbidities, antifungal therapy, and mortality data were recorded. Multimorbidity was measured with the Charlson Comorbidity score (CCS) derived from the medical record data [14].

Serum GM-ELISA and GM-LFA levels were measured from all patients twice a week, NBL GM-ELISA, GM-LFA levels, and fungal culture from intubated patients once a week regularly until clinician's CAPA diagnosis, ICU discharge, or mortality. These samples were collected from patients if at least seven days had passed since the first SARS-CoV-2 PCR positivity. If this criterion was met, the samples were taken within 48 h of ICU admission. A closed suction system collected NBL by giving 20 mL sterile saline in intubated patients, as previously described [15]. Collected serum and NBL samples were stored at −80 °C for less than six months. Patients were diagnosed with CAPA according to ECMM/ISHAM criteria and without considering GM-LFA results [5]. The gold standard method for diagnosis was histopathology, but there was no patient who has a proven diagnosis. The clinicians continued their routine follow-up;

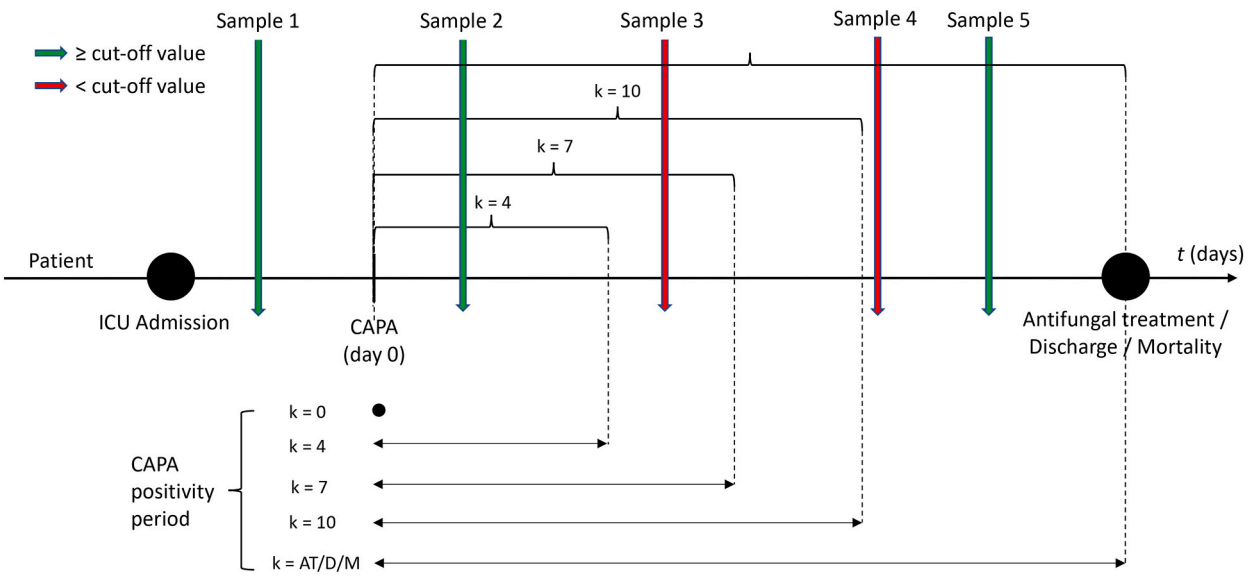

Fig. 1. Assessment of LFA results based on different case definitions. ICU: intensive care unit, CAPA: COVID-19 associated pulmonary aspergillosis. AT/D/M: Antifungal treatment/Discharge/Mortality. For example, if k = 4, Samples 1 and 5 were considered false positive, Sample 2 was true positive, and Sample 3 and 4 were true negative test results. If k = 7, Sample 3 was started to be considered false negative.

they evaluated the patients and tested for CAPA whenever they suspected fungal infection, and they did not have any information about collected samples during the study period. Patients who were diagnosed by healthcare professionals and their clinical samples were also included in the analysis.

2.2. Aspergillus Galactomannan Lateral Flow Assay

GM antigen detection, fungal culture, and direct examination of respiratory specimens were explained in our previous study in detail [13]. The sōna *Aspergillus* GM-LFA (IMMY Diagnostics, Norman, OK, USA) was performed on serum and NBL specimens according to the manufacturer's instructions [16. Briefly, 300 µl serum or NBL specimen was placed in a screw cap, heat-resistant microcentrifuge tube, and 100 µl sample pre-treatment buffer was added. The tube was vortexed and incubated in a heat block at 120 °C for 6–8 min, followed by immediate centrifugation at 10,000–14,000 g. Eighty µl of the supernatant was added onto 40 µl of running buffer in a separate clean tube and mixed. The test strip was placed in the tube, which was incubated at room temperature for 30 min. The result obtained using Cube Reader (IMMY Diagnostics, Norman, OK, USA) within 10 min was recorded. Positive and negative control was used for each run.

2.3. Defining case definitions for CAPA patients

In the CAPA group, the day of diagnostic criteria were met was considered day 0. We set different time intervals (k-days) as case definitions, as it can be debatable how long serum and NBL GM-LFA tests could remain positive after CAPA diagnosis (Fig. 1). We calculated the performance metrics and AUC values based on different k-days accordingly.

The cut-off values of 0.5 for serum and 1.0 for NBL were used to calculate the sensitivity and specificity for GM-LFA [5]. Since repetitive samples were taken from patients, each result was evaluated as positive or negative based on the time interval the patients were diagnosed with CAPA or not, as schematized. If the sampling day was after the k-period for the patient, this sample's result was not included in the analysis. A final analysis was done for serum GM-LFA results by calculating the k value until discharge, death, or antifungal treatment. In the non-CAPA group, all serum and NBL GM-LFA results above cut-off values were considered false positive, and below cut-off values were true negative.

2.4. Joint model application for serum GM-ELISA and serum GM-LFA

CAPA is associated with high mortality [2,13]. Therefore, we used the joint model framework to assess the association of longitudinal levels of serum GM-LFA with mortality and compared these levels with longitudinal levels of serum GM-ELISA. Detailed information about the framework was provided in the supplementary file.

2.5. Statistical analysis

Mean and standard deviation for normally distributed data, median (interquartile range [IQR]) for non-normally distributed data and percentage for categorical variables were used. Continuous variables were compared using Mann–Whitney *U* test, Fisher's exact test, and the chi-squared test for categorical comparisons. Receiver operating characteristics (ROC) analysis was performed for serum GM-LFA and NBL GM-LFA. AUC values were presented, including 95 % confidence intervals (CI) for the CAPA diagnosis. The pairwise correlation between serum GM-ELISA, serum GM-LFA, NBL GM-ELISA, and NBL GM-LFA was calculated using Spearman correlation analysis.

We used the Pandas library (version 1.25.3) to manipulate and analyze the data, the Sci-kit Learn library (version 1.0.2) to draw ROC curves, calculate AUC scores, and build Kaplan-Meier Estimator for survival analysis, and the SciPy library (version 1.7.3) to calculate Spearman's correlation and other statistical tests in Python 3.7. Confidence intervals for test statistics were exact Clopper-

Table 1
Mycological test results of patients with CAPA diagnosis.

n	NBL Aspergillus culture	NBL GM-ELISA index >4.5	NBL GM-ELISA index >1.2 (x2)	Serum GM-ELISA ≥0.5	Diagnosis
17				x	Probable
4	x			x	Probable
3	x	x		x	Probable
2		x		x	Probable
1	x		x	x	Probable
1	x		x		Possible
1			x		Possible
4	x	x			Possible
6	x				Possible
2		x			Possible

CAPA: COVID-19 associated pulmonary aspergillosis, NBL: nondirected bronchial lavage, GM: galactomannan, 27 patients were in probable, and 14 were in possible groups according to ECMM/ISHAM criteria.

Pearson confidence intervals. Confidence intervals for AUC values were calculated using bootstrapping (number of bootstraps = 1000). A two-sided p-value <0.05 was considered significant. The proportional hazard assumption was checked statistically using the Schoenfeld Residuals Test for the Cox PH model. The package JMbayes2 (version 0.3-0) was used for the joint model application in R 4.2.1 [17].

This study was approved by the Institutional Review Board of our hospital (Date: November 17, 2020, GO 20/1121).

3. Results

A total of 207 patients were evaluated for analysis (Fig. S1). The mean age was 66 ± 13.3 years, and 40 % were female. The mean BMI was 27.9 ± 6.5 kg/$m^{2,}$ and the median for CCS was 1 (0–3). 41 (20 %) patients were diagnosed with CAPA. The mortality rates for 28 and 60 days were 28.5 % and 38.2 %, respectively.

A total of 750 blood samples and 170 NBL samples were collected from 207 patients during their ICU stay (Table S1). Which

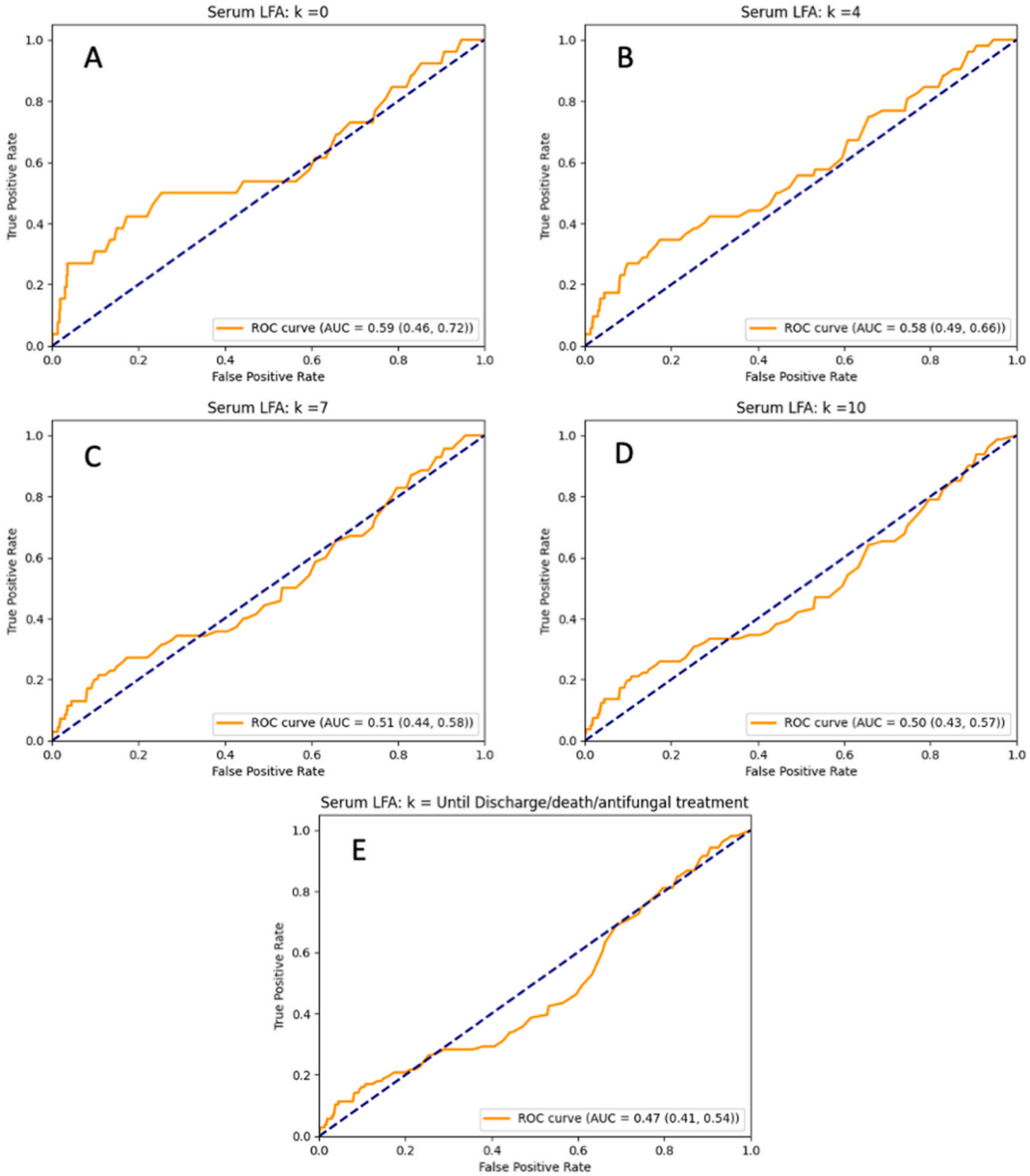

Fig. 2. Receiver operating characteristics (ROC) for serum galactomannan lateral flow assay in different case definition periods; A: ROC for the day of CAPA diagnosis, B: ROC for the tests within 4 days of CAPA, C: ROC for the tests within 7 days of CAPA, D: ROC for the tests within 10 days of CAPA, E: ROC for the tests performed until discharge/death/antifungal treatment.

microbiological tests were positive in patients diagnosed with probable and possible CAPA are shown in Table 1. On the day of CAPA diagnosis, serum GM-LFA with a cut-off 0.5 had a sensitivity of 42 % (95 % CI: 23–63), specificity of 82 % (95 % CI: 78–84); while NBL GM-LFA with a cut-off 1 had a sensitivity of 73 % (95 % CI: 45–92), specificity of 85 % (95 % CI: 76–91) for CAPA diagnosis (Figs. 2–3, Tables S2–S7). Sensitivity was at the highest point on day 0 and started to decrease through the following days in both sample groups.

There were no patients on antifungal prophylaxis during the study period. Colonization with *Candida* spp. was detected in 21 (56.8 %) among 37 NBL GM-LFA positive samples and in 64 (48.1 %) among 133 NBL GM-LFA negative samples ($p=0.45$).

In univariate joint model analysis, both CCS and logarithmic transformations of serum biomarkers were found to be significant ($p < 0.05$) (Table 2). In log(serum GM-LFA) univariate analysis, an increase in CCS by one unit was associated with a hazard ratio of exp (0.18) = 1.19, and doubling in serum GM-LFA levels results in a 2.05-fold increased risk of death ($2^{1.04} = 2.05$). In log(serum GM-ELISA) univariate analysis, CCS's hazard ratio was found as exp (0.13) = 1.13, and doubling in serum GM-ELISA levels resulted in a 4.89-fold increased risk of death ($2^{2.29} = 4.89$). In multivariate analysis, only CCS and serum GM-ELISA levels were significant (Table 3). The results revealed that an increase in CCS by one unit increased the relative risk of death by 14 % (exp (0.13) = 1.14) (%95 CI: 1.03–1.27) and doubling in serum GM-ELISA levels results in a 3.07-fold (%95 CI: 1.14–12.91) increased risk of death ($2^{1.62} = 3.07$).

4. Discussion

In this study, we analyzed serum and NBL GM-LFA diagnostic performance with different case definitions. Our aim was to evaluate the robustness of these biomarkers after CAPA diagnosis. The highest sensitivity and specificity values were detected on day 0. Sensitivity decreased in the following days in both serum and NBL sample groups. In univariate analysis, doubling in serum GM-LFA levels resulted in a 2.05-fold increased risk, and doubling in serum GM-ELISA levels resulted in a 4.89-fold increased risk of death. We also found that according to multivariate joint model analysis, doubling in serum GM-ELISA levels caused a 3.07-fold increased risk of death after adjusting age, BMI, sex, and serum LFA in all ICU patients.

Studies evaluating the performance of GM-LFA in CAPA patients have reported different results. In a study by Autier et al., the sensitivity of GM-LFA was 80 %, and specificity was 88 % for NBL using IMMY *Aspergillus* LFA at the 1.0 cut-off (17), as well as, the sensitivity and specificity of serum LFA were 20 % and 93 %, respectively, at the 0.5 ODI cut-off. A recently published study that

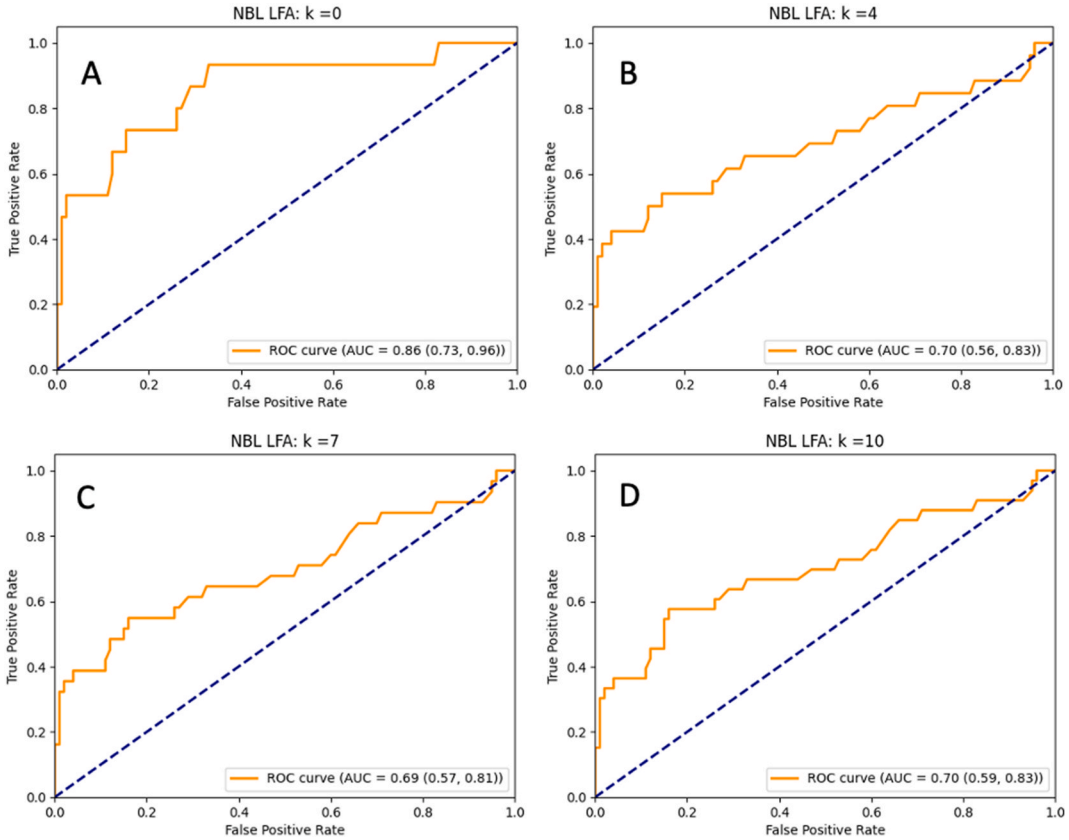

Fig. 3. Receiver operating characteristics (ROC) for nondirected bronchial lavage galactomannan lateral flow assay in different case definition periods; A: ROC for the day of CAPA diagnosis, B: ROC for the tests within 4 days of CAPA, C: ROC for the tests within 7 days of CAPA, D: ROC for the tests within 10 days of CAPA.

Table 2
Univariate joint models: the association of longitudinal levels of serum GM-LFA, and serum ELISA-LFA and clinical parameters with mortality.

Joint Model	Standardized coefficients (95 % CI)	p-value
log (Serum LFA)		
Number of groups: 203		
Number of events: 77 (37.9 %)		
Number of observations: 749		
Linear mixed submodel		
Intercept	−1.29 (−1.43, −1.16)	<0.001
Time (per day)	0.00 (−0.01, 0.01)	0.46
Cox regression submodel		
Age	0.03 (0.00, 0.05)	**0.03**
BMI	−0.02 (−0.08, 0.04)	0.65
Sex	−0.37 (−0.88, 0.11)	0.15
CCS	0.17 (0.08, 0.28)	**<0.001**
log (Serum LFA)	1.04 (0.11, 2.25)	**0.03**
log (serum ELISA)		
Number of groups: 203		
Number of events: 77 (37.9 %)		
Number of observations: 908		
Linear mixed submodel		
Intercept	−1.82 (−1.92, −1.73)	<0.001
Time (per day)	0.00 (0.00, 0.01)	0.34
Cox regression submodel		
Age	0.02 (0.00, 0.05)	0.07
BMI	−0.01 (−0.07, 0.04)	0.89
Sex	−0.24 (−0.76, 0.30)	0.37
CCS	0.13 (0.03, 0.23)	**0.02**
log (Serum ELISA)	2.30 (0.54, 4.79)	**0.01**

LFA: lateral flow assay, CI: confidence interval, BMI: body mass index, CCS: Charlson comorbidity score.

Table 3
Multivariate joint model: the association of longitudinal levels of logarithmic transformation of serum GM-LFA, and serum ELISA-LFA and clinical parameters with mortality.

Joint Model	Standardized coefficients (95 % CI)	p-value
Number of groups: 203		
Number of events: 77 (37.9 %)		
Number of observations per biomarker: 739		
Linear mixed submodel (log (Blood LFA)		
Intercept	−1.30 (−1.44, −1.15)	<0.001
Time (per day)	0.00 (−0.01, 0.01)	0.51
Linear mixed submodel (log (Blood ELISA)		
Intercept	−1.88 (−1.98, −1.78)	<0.001
Time (per day)	0.01 (0.00, 0.01)	0.14
Cox regression submodel		
Age	0.02 (0.00, 0.05)	0.06
BMI	0.00 (−0.06, 0.05)	0.98
Sex	−0.21 (−0.75, 0.34)	0.44
CCS	0.13 (0.03, 0.24)	**0.01**
log (Serum LFA)	0.69 (−0.39, 1.87)	0.20
log (Serum ELISA)	1.62 (0.14, 3.70)	**0.03**

LFA: lateral flow assay, CI: confidence interval, BMI: body mass index, CCS: Charlson comorbidity score.

compared LFA (IMMY *Aspergillus* LFA) with GM-ELISA and BAL culture in patients diagnosed with CAPA according to ECMM criteria showed that serum GM-LFA sensitivity was 56 %, specificity 94 %, BAL LFA sensitivity was 60 %, and specificity 88 %. For CAPA diagnosis, the predictive performance of LFA and GM-ELISA in serum and BAL samples were similar [18]. In another study evaluating the accuracy of LFA on tracheal aspirate, a sensitivity of 60 % and specificity of 72.6 % were reported [19]. These two latter studies were performed on patients with invasive mechanical ventilation only. In our study, sensitivity and specificity values for the day of CAPA diagnosis ($k = 0$) were consistent with the literature, and these values decreased in the following days. As far as we know, our study is unique in the literature in terms of assessing the robustness of GM-LFA results in CAPA patients. In some tests, sensitivity and specificity values may be highest during the period when the clinical signs of the disease are most intense. The most well-known example for this is the variability in blood culture results in bacteremia. Repeated blood culture sampling is recommended because bacteraemia occurs intermittently [20]. In terms of herpes simplex virus infection, to detect the virus by culture or immunoassay is highest when the lesions are in the vesicular stage [21]. There is no information yet regarding the sensitivity and specificity results of

patients in the period after diagnosis in screening studies on LFA. Although serum GM-LFA positivity is a criterion for probable CAPA according to ECMM/ISHAM criteria, its diagnostic value was not found to be as good as NBL GM-LFA.

Previous studies have generally evaluated the accuracy of GM-LFA on clinical suspicion of CAPA and compared GM-LFA and GM-ELISA in patients diagnosed with CAPA. In just one study, GM-LFA was performed as a screening test in critically ill patients [22]. In that study, there was a good agreement between GM-ELISA and GM-LFA at 92 %. Probable CAPA incidence was 10.2 % (19/185 patients) if GM-LFA was used as a mycological criterion and 9 % (9/100) if GM-ELISA was used as a mycological criterion. The authors reported a similar mortality rate for CAPA and non-CAPA groups which was quite different from the current literature. In our cohort, mortality was much higher in the CAPA group (67.4 % vs 29.4 %) [13]. To our knowledge, no study is investigating the association of longitudinal measures of GM-ELISA and GM-LFA with mortality in COVID-19 patients. Joint modeling of longitudinal data and survival analysis was a beneficial method for producing more accurate dynamic prediction studies in different clinical entities [23]. In serial measurements, doubling serum GM-ELISA and GM-LFA levels were found to be associated with higher mortality. However, after adjusting for age, sex, and BMI in multivariate joint model analysis, serum GM-LFA did not show the same effect. Three times folded mortality risk was found in serum GM-ELISA doubling. This finding provides important evidence for the usage of serum GM-ELISA as a screening tool in COVID-19 patients who were hospitalized in ICU units because of respiratory failure.

We found a very weak-to-weak correlation across biomarkers. As reasons for this, firstly, we sampled these biomarkers from all patients regardless of clinical suspicion of CAPA which made it difficult to compare our results with others in the literature. Second, discrepant results were also reported previously. A study comparing GM-ELISA and GM-LFA in different periods for IPA showed that previous exposure to antibiotics could influence the lateral flow device results, leading to discrepancies in results [24]. Although we did not record antibiotic use in our patient population in the ICU, it is plausible to consider this factor might be effective. On the other hand, false-positive results for BAL GM-LFA results have also been reported. In a study by Farmakiotis et al., positive predictive value (PPV) changed according to the pre-test probability with worse PPVs in non-hematological patients [25]. Colonization with *Candida* species in the respiratory tract was shown as a potential reason for galactomannan positivity in nonhematological patients as well [26]. In our cohort, we did not detect a statistically significant effect of *Candida* colonization on NBL GM-LFA results.

As limitations; bronchoscopy is the gold standard for IPA diagnosis; NBL might underestimate our positive results. In addition, NBL GM-ELISA and NBL GM-LFA test results were not analyzed in the joint model because NBL samples were only collected from intubated patients, which would lead to biased results that were difficult to compare with serum samples.

In conclusion, GM-LFA, particularly in NBL samples, seems to be a reliable method for CAPA diagnosis. Diagnostic performances of both serum and NBL GM-LFA tend to decrease in the following days after CAPA diagnosis. For detecting patients with higher risk of mortality, longitudinal measurement of serum GM-ELISA can be useful.

Data availability statement

Data will be available reasonable request.

CRediT authorship contribution statement

Berrin Er: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ahmet Gorkem Er:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dolunay Gulmez:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Taha Koray Sahin:** Writing – review & editing, Resources, Project administration, Data curation, Conceptualization. **Gökhan Metan:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Zeynep Saribas:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Sevtap Arikan-Akdagli:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Omrum Uzun:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21721.

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